Marta Prado[1]
Carlos M. Franco[1]
Cristina A. Fente[1]
Alberto Cepeda[1]
Beatriz I. Vázquez[2]
Jorge Barros-Velázquez[2]

[1]Area de Nutrición
y Bromatología
[2]Area de Tecnología
de Alimentos,
Departamento
de Química Analítica,
Nutrición y Bromatología,
Facultad de Veterinaria,
Universidad de Santiago
de Compostela,
Lugo, Spain

Comparison of extraction methods for the recovery, amplification and species-specific analysis of DNA from bone and bone meals

We report the effect of several parameters on the efficiency of recovery of DNA from animal bones. The effects of preheating the samples (at either 607C or 1007C) at different intervals (from 1 h to overnight) in different media (water, 0.5 M ethylenediaminetetraacetic acid (EDTA), or 0.5 M EDTA 1 0.05% sodium dodecyl sulfate (SDS) were investigated. The effect of slight (5 min) or intense (30 min) pretreatments with ultrasound was also evaluated. Several different treatments with proteinase K (ranging from 200 to 800 mg, and lasting from 1 to 3 h) at 657C were also considered. Addition- ally, two different DNA extraction methods (based on silica resins and purification col- umns, respectively) were evaluated. The recovery of DNA from the samples was 40% higher when the bones were preheated in 0.5 M EDTA at 607C for 1 h, this being fol- lowed by treatment with 800 mg of proteinase K for 3 h. The DNA thus obtained was successfully amplified by polymerase chain reaction (PCR) using a set of primers specific to a 359 bp region of the mitochondrial cytochrome *b* gene, and the species of origin were identified by visualizing the restriction fragment length polymorphism (RFLP) with the endonucleases *Pal*I and *Mbo*I.

Keywords: Bone meals / DNA recovery / Extraction methods / Polymerase chain reaction

Correspondence: Prof. Jorge Barros-Velázquez, Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Santiago de Compostela, E-27002 Lugo, Spain **E-mail:** jbarros@lugo.usc.es **Fax:** 134-982-252195

1 Introduction

The commercialization of animal feeds infected by prions has proved to be the main cause of transmission of bovine spongiform encephalopathy (BSE) [1], a syndrome that, in addition to its negative implications in public health [2, 3], has caused severe economic losses for the meat sector of the European Union (EU) [4]. This situation has led the European Commission (EC) to extend the pro- hibition of including any material of animal origin in meals destined for animal consumption. Thus, EU-enforced regulations have established the compulsory examination of animal meals in an effort to guarantee that these pro- ducts do not include any prohibited material, basically the remains of animal cells, with a view to preventing the transmission of BSE in Europe.

The methodology currently employed for the control of animal meals is based on the microscopic detection of animal materials, a technique that is especially useful for the detection of bone meals and other anatomical parts of remarkable hardness, and has now become the method officially used in the EU (Directive 98/88/EC). This micro- scopic technique is characterized by its high sensitivity in the specific detection of bones from land animals, although it also allows the detection of materials from marine species. Nonetheless, this technique may not be suitable for the detection of other animal materials, such as blood or specific risk materials (SRM), thus complicat- ing the successful traceability of such animal meals, with the ensuing negative implications for animal safety. With a view to overcoming the limitations of microscopy in the detection of blood and other soft parts of land animals, PCR and immunoassay have been proposed to be accu- rate methods for the successful traceability of many types of animal meals [5]. It is clear why other techniques based on protein analysis (isoelectric focusing, native poly- acrylamide electrophoresis, SDS-polyacrylamide electro- phoresis, chromatography, 2-DE) fail to detect specific tissues, because they depend on gene expression and hence vary in their accuracy, depending on the type of tissue included in the meal. Besides the fact that most protein-based detection methods would not be accurate for the detection of bone material in animal meals, it should also be stressed that such protein-based methods would not be suitable for processed materials because most of the target proteins are degraded very rapidly under heat conditions [6–8]. Therefore, and although some thermostable proteins in fact have been proposed for the authentication of species in meat [9] and fish pro- ducts [10], such methods have not been tested in animal

feeds. Likewise, some immunological techniques have also been used for species verification [11, 12] but, because they rely on antibodies, these methods often involve cross-reactions that may lead to ambiguous results in the case of closely related species and mixtures of species [13].

In contrast to these limitations, over the past decade DNA-based detection methods such as DNA hybridiza- tion, DNA amplification and DNA sequencing have been successfully employed for the species-specific characterization of meat and other food products [14–18]. These methods have recently been proposed for the species-specific detection of meat-containing animal meals, because of their high sensitivity and the fact that DNA is a thermostable molecule, able to resist severe heat treatments such as those involved in the manufacturing of animal meals. While the extraction of DNA from meat has been widely reported [15, 16, 18–20], considerable difficulties may arise in the recovery of amplifiable DNA from bone samples, a drawback that would limit the usefulness of PCR in the tracing of bone meals [21, 22]. Thus, although several reports on the recovery and successful PCR-amplification of DNA from bone samples have indeed been made they correspond to forensic [22, 23], archaeological [21, 24] or anthropological studies [25, 26]. Some of these protocols are based on the evaluation of several extraction buffers coupled with phenol-chloroform extraction [27, 28] and/or treatment with EDTA and Tris-HCl-buffered solution [28]. After the extraction step, the DNA-containing aqueous phase is usually concentrated by ethanol or isopropanol precipitation [28, 29], or using microconcentration systems [27, 28, 30]. Further DNA purification normally involves silica parti- cles, which have a high binding capacity for DNA mole- cules [26], the DNA being recovered from this matrix by specific elution.

Unlike the protocols described for forensic, archaeological and anthropological studies, to date little attention has been paid to the development of a standardized protocol for the recovery, amplification and species-spe- cific characterization of bone cells in animal meals. A DNA extraction protocol of this kind should be able to purify trace amounts of DNA while at the same time removing potential inhibitors of PCR [30]. Here, we report the effect of several parameters on the efficiency of DNA recovery from bone samples of land animals within the general context of exploring ways of preventing the transmission of BSE. The evaluation of several pretreat- ments and two extraction methods for the species-spe- cific detection of DNA from bone samples, home-made bone meals and industrial meat and bone meals (MBMs) is discussed.

2 Materials and methods

2.1 Preparation of bone samples and artificial contamination of wheat flour

Bone samples from pigs (*Sus scrofa*) were scraped with sterile surgical blades and bone fragments were collected in a sterile Petri dish placed underneath the bone to avoid any contamination. Portions of 0.24–0.25 g of this bone powder were placed in sterile 2 mL microtubes for evaluating each of the different pretreatments considered (Table 1). When required, home-made bone meals were prepared by mixing commercial wheat flour with bone powder at the following ratios: 100/0, 99.9/0.1, 99.5/0.5, 99/1, 97.5/2.5, and 90/10, respectively. Additionally, industrial MBMs prepared at high temperatures during 1999 by a local factory were studied. When required, commercial wheat flour was also contaminated with MBM powder at the following ratios: 100/0, 99.9/0.1, 99.5/0.5, 99/1, 97.5/2.5, and 90/10, respectively.

Table 1. Pretreatments evaluated in the optimization of DNA recovery from bone samples

Extracting reagent	Temperature/time of incubation in extracting reagent	Treatment with proteinase K[a)] (10 mg/mL)	DNA concentration (ng/mL)
Milli-Q water	607C/16 h	20 mL/1 h	35.3
Milli-Q water	607C/16 h	80 mL/1 h	6.3
Milli-Q water	607C/16 h	80 mL/3 h	34.2
0.5 M EDTA	607C/16 h	20 mL/1 h	22.8
0.5 M EDTA	607C/16 h	80 mL/1 h	92.0
0.5 M EDTA	607C/16 h	80 mL/3 h	188.4
0.5 M EDTA	607C/1 h	20 mL/1 h	97.0
0.5 M EDTA	607C/1 h	80 mL/1 h	77.7
0.5 M EDTA	607C/1 h	80 mL/3 h	232.7
0.5 M EDTA	1007C/1 h	20 mL/1 h	42.2
0.5 M EDTA	1007C/1 h	80 mL/1 h	58.3
0.5 M EDTA	1007C/1 h	80 mL/3 h	93.3
0.5 M EDTA1 0.05% SDS	1007C/1 h	20 mL/1 h	114.1
0.5 M EDTA1 0.05% SDS	1007C/1 h	80 mL/1 h	117.0
0.5 M EDTA1 0.05% SDS	1007C/1 h	80 mL/3 h	119.1
Milli-Q water	1007C/1 h	20 mL/1 h	58.4
Milli-Q water	1007C/1 h	80 mL/1 h	68.4
Milli-Q water	1007C/1 h	80 mL/3 h	70.5

a) After pretreatment with each extracting reagent at the indicated temperatures and times, the samples were centrifuged (13 000 rpm/15 min) and the pellets were resuspended in lysis buffer (Qiagen DNeasy minikit) and treated with proteinase K.

2.2 Microscopic detection of osteocytes in home-made bone meals and industrial MBMs

The official microscopic method was used to detect the presence of bone residues in the home-made bone meals and in the MBMs. Briefly, samples were initially ground to a final size of 1 mm. Ten grams was transferred to a decanting funnel together with 100 mL of tetrachloroethylene (density = 1.62 g/mL; Merck, Darmstadt, Germany) and this mixture was shaken vigorously for at least 10 s. After decanting for 5–10 min, the mixture was shaken one more time and allowed to stand again for at least 45 min. Next, 7 mL of a liquid phase with a density of 2.2 g/mL was added. This liquid phase was obtained by mixing tetrachloroethylene (density = 1.62 g/mL; Merck) and tetrabromoethane (density = 2.96 g/mL; Prolabo, Briare, France) at a ratio of 57/43 (v/v), respectively. Three fractions were obtained: a first fraction, with a density lower than 1.62 g/mL, containing mainly vegetable remains; a second fraction, with a density between 1.62 and 2.2 g/mL, containing all the bones but also some mineral and vegetable remains, and a third fraction, with a density higher than 2.2 g/mL, containing most of the mineral remains. The second fraction, with a density between 1.62 and 2.2 g/mL, was retrieved and placed inside a previously weighed test tube. Then, 1 mL of sodium hypochlorite (Euroquímica, S.A., Toledo, Spain) was added to the tube. After 10 min, the tube was filled with water, allowed to stand for 2–3 min and the water-containing particles in suspension – basically vegetable remains – were eliminated. This step was repeated three times with 10 mL of water each time. Next, two to ten drops of alizarin red reagent were added (alizarin red was prepared as follows: 0.2 g of alizarin red S (Prolabo) were mixed with 2.5 mL of 1 N HCl (Merck), and distilled water to a final volume of 100 mL). The stained material was washed twice with 5 mL of alcohol and once with 5 mL of acetone (Merck). After each washing step, the stained material was allowed to stand for 1 min until the solvent was eliminated. Finally, the stained material was dried in an oven (P-Selecta, Barcelona, Spain) for 10–20 min, and the weight of the solid fraction was determined. The stained solid fraction was observed under a phase-contrast microscope (model CH-2; Olympus, Barcelona, Spain) and the characteristic bone fragments were detected and determined.

2.3 DNA extraction

2.3.1 Cellular lysis pretreatments

As observed in Table 1, the effect of preheating the sample in different extracting reagents, at different temperatures and times, and treatments of different intensity with

Table 2. Effect of ultrasound on DNA recovery from bones

Time of treatment with ultrasound	Extracting conditions	Proteinase K (10 mg/mL)	DNA recovered (ng/mL)
0 min	0.5 M EDTA/607C/1 h	80 mL/3 h	232.7
5 min	0.5 M EDTA/607C/1 h	80 mL/3 h	116.2
30 min	0.5 M EDTA/607C/1 h	80 mL/3 h	72.7

The samples were resuspended in 0.5 M EDTA and subjected to treatment with ultrasound at room temperature, as stated above. Once the ultrasound pretreatment had been completed, the samples were heated and kept at 607C for 1 h, centrifuged (13 000 rpm/15 min); the pellets were resuspended in lysis buffer (Qiagen DNeasy minikit) and treated with proteinase K.

Table 3. Effect of supernatant and pellet processing on DNA recovery from bone meals

Extracting conditions	Proteinase K (10 mg/mL)	Type of sample processing	DNA recovered[a)] (ng/mL)
0.5 M EDTA/607C/1 h	80 mL/3 h	Supernatant	54.9
0.5 M EDTA/607C/1 h	80 mL/3 h	Pellet	117.8
0.5 M EDTA/607C/1 h	80 mL/3 h	Supernatant1 pellet	239.4
No pretreatment (blank assay)	80 mL/3 h	Supernatant1 pellet	170.3

a) Results are expressed in terms of their efficiency as compared with the assay in which DNA was recovered from the bone pellet.

proteinase K were evaluated. The effect of ultrasound on the recovery of amplifiable DNA was also evaluated (Table 2) by means of an ultrasound equipment (Raypa, Model UCI-200, Barcelona, Spain) working at 35 kHz. Finally, once the optimal pretreatment had been identified, the effect of combining the pellet and supernatant phases of the pretreated extract was evaluated (Table 3). Two commercial DNA extraction kits were used in this study. Both kits include two phases: (i) a lysis phase, in which tissues and cell membranes are lysed and removed, and (ii) a purification phase, in which proteins, RNA, carbohydrates and other subcellular material are removed and DNA is recovered specifically.

2.3.2 DNA purification method based on capture with silica resins

0.1–0.2 g of the samples was placed in a microtube previously sterilized in an autoclave (Raypa, AE 75 TIC Model, Barcelona, Spain), after which 1 mL of B buffer

(Biotools, Madrid, Spain), 15 mL of b-mercaptoethanol (Sigma, St. Louis, MO, USA) and 20 mL of proteinase K (10 mg/mL; Sigma) were added. Once all the reagents had been added they were suitably mixed and incubated under shaking in a thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany) at 657C for 2 h. Cellular debris was removed by spinning at 13 000 rpm for 15 min (Centrifuge 5415 D; Eppendorf), the supernatant being recovered and transferred to a new tube. Then, 100 mg of RNase A (10 mg/mL; Sigma) was added, followed by incubation at 377C for 1 h in the thermomixer. Following this, 0.7 volumes of chloroform (Merck)/isoamylalcohol (Probus, Barcelona, Spain) (24/1) were added, the mixture was vigorously vortexed and centrifuged at 13 000 rpm for 10 min, and the DNA-containing aqueous upper phase was recovered by gentle pipetting and transferred to a new vial. DNA purification was carried out by adding 1 volume of Solution I (Biotools, Madrid, Spain) and 80 mL of silica matrix (Biotools) and mixing thoroughly by gentle pipetting. After 10 min of incubation at room temperature, mixing occasionally by inverting the tubes up and down, the mixture was centrifuged at 10 000 rpm for 3 min and the supernatant discarded. Then, 750 mL of Wash Solution (Biotools) was added to the pellet, mixed by gentle pipetting, centrifuged at 10 000 rpm for 3 min, and the supernatant discarded. This step was repeated to ensure the removal of any potentially inhibitory substances, and the pellets were recovered by spinning at 13 000 rpm for 30 s. After draining off any residual Wash Solution, the pellet was dried at 657C for 2 min and resuspended in 100 mL of 10 mM Tris-HCl (pH 8.0), incubated at 657C for 10 min and centrifuged at 13 000 rpm for 5 min. Finally, 80 mL of the supernatant was transferred to a new vial, centrifuged again to ensure that no matrix trace was present, and the supernatant was stored at –207C until analysis.

2.3.3 DNA purification method based on columns

The DNeasy Plant minikit (Qiagen, Washington, DC, USA) was also used for the extraction of DNA from 100 mg of sample. In this protocol, the bone material was first mechanically disrupted and then lysed by the addition of Lysis Buffer (Qiagen), followed by incubation at 657C. RNase is included in the commercial Lysis Buffer provided by the kit to remove RNA molecules from the DNA extract. Proteins and polysaccharides were removed by salt precipitation. Precipitates and cellular debris were removed by spinning through the QIAshredder filtration unit and the cleared lysate was transferred to a new tube, where a binding buffer and ethanol were added to facilitate the binding of DNA to the DNeasy membrane. The DNA extract was then applied to a DNeasy column and centrifuged briefly. DNA bound to the membrane while the other compounds were removed through subsequent washing steps. Purified DNA was finally eluted from the membrane and then stored at –207C until analysis.

2.4 Determination of DNA concentrations in the extracts

This was carried out by measuring the fluorescence developed by mixtures of the purified DNA extract and Hoechst 33258 reagent (Sigma) in a LS 50B fluorimeter (Perkin Elmer, Wellesley, MA, USA). Hoechst 33258 reagent is a bis-benzimide that acts as a DNA intercalator, exciting in the near UV (350 nm) and emitting in the blue region of the visible spectrum (450 nm). The sensitivity of the Hoechst 33258 assay is *ca.* 5 ng/mL. 100 mL of a freshly prepared Hoechst solution was employed for each set of determinations. The Hoechst solution was prepared by mixing 10 mL of 106 TNE, 10 mL of Hoechst 33258 reagent (1 mg/mL) and 90 mL of Milli-Q water. 106 TNE was prepared by mixing 12.11 g of Tris-base (Merck), 3.72 g of EDTA disodium salt-dihydrate (Calbiochem, San Diego, CA, USA), 116.89 g of sodium chloride (Merck) and then ultrapure Milli-Q water up to a final volume of 1000 mL, adjusting pH to 7.4 with concentrated HCl. Two standard curves were made in order to verify the lin- earity of the assay within a particular concentration range. Both standard curves were constructed on calf thymus DNA (Sigma) standard solutions. Calf thymus DNA stan- dard solutions were prepared in the following concentra- tion ranges: (i) 10–150 ng/mL; and (ii) 100–1000 ng/mL. Dilutions were made directly in the cuvettes by mixing the Hoechst solution with the standard DNA. Standard curves were constructed with EXCEL software from Microsoft. Correlation coefficients (r^2) higher than 0.99 were achieved in all cases.

2.5 PCR-based detection of animal and plant DNA

Amplification of purified DNA was carried out in a final volume of 50 mL, contained in 0.5 mL microcentrifuge tubes. Each amplification assay contained 100 ng of template DNA, 15 mL of Master Mix (Biotools) – this including accurate concentrations of reaction buffer, dNTPs and primers – 2 mL of magnesium chloride (Biotools), 1 mL of *Taq* DNA polymerase (Biotools), and PCR water (Genaxis, Montigny le Bretonneaux, France) to achieve a final volume of 50 mL. Oligonucleotide primers were the forward primer CYT b1 (5'-CCATCCAACATCT CAGCATGATGAAA-3') and reverse CYT b2 (5'-GCCCCT CAGAATGATATTTGTCCTCA-3'), described by Meyer *et*

al. [17] for the detection of DNA from land animals on the basis of the amplification of a well-conserved 359 bp DNA fragment of the mitochondrial cytochrome *b* gene. Alternatively, a Duplex-Master Mix (Biotools) was employed, including this set of primers and another set of primers specific to plant DNA. Thus, the Duplex-Master Mix combined two set of primers, one specific to animal DNA (visualized as a 359 bp DNA fragment) and a second set of primers, which allows the amplification of a smaller DNA fragment characteristic of plants (visualized as a 190 bp DNA fragment) and which acts as a positive con- trol of the PCR assay in MBMs. 2 mL of $MgCl_2$ solution (Biotools) and 1 mL of *Taq* polymerase (Biotools) were used in each amplification assay. The following PCR protocol was employed: an initial denaturation step at 947C for 1 min 30 s was followed by 45 cycles of denaturation (947C for 10 s), annealing (557C for 30 s), and extension (727C for 40 s). A final extension step at 72ºC/15 min was considered. All PCR assays were carried out on a Progene thermal cycler (Progene, Techne, Princeton, NY, USA). DNA amplification products were processed in 2.5% horizontal agarose (MS-8, Pronadisa, Madrid, Spain) gels in 16 TAE (Tris-acetate-EDTA) buffer, nucleic acid sizes being estimated by comparison with a ladder consisting of a *Msp*I-digest of plasmid pUC18 (Sigma). Gels included 0.5 mg/mL of ethidium bromide (Merck) for visualization purposes at 254 nm. The gels were run at 100 V. Gels corresponding to the RFLPs were prepared with 4% agarose and run at 50 V. When required, species identification was carried out by estimating the number and size of the DNA fragments obtained by cleaving the 359 bp amplifica- tion fragment with the endonucleases *Pal*I and *Mbo*I, which allowed the identification of bovine, porcine, sheep, goat, chicken, goose, turkey, deer, kangaroo, buffalo and several other animal species of interest.

3 Results and discussion

3.1 Effect of several pretreatments on the recovery of DNA from bones

As mentioned above, two commercial methods were used in this study. One of them (Biotools) was based on DNA purification with resins. The other method (Qiagen) was based on DNA purification by capture columns. Both methods allowed the recovery of amplifiable DNA from bone samples (Fig. 1A). Additionally, both extraction methods permitted successful detection of the 359 bp PCR product in industrial MBMs, as can be observed in Fig. 1B. However, the DNeasy kit from Qiagen was selected because it afforded a simpler and faster DNA purification, also avoiding the tedious extraction of samples with chloroform/isoamylic alcohol, with no significant decrease in DNA recovery.

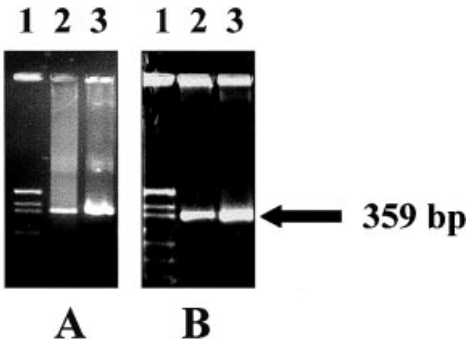

Figure 1. Agarose gel electrophoresis of the 359 bp PCR product obtained after extraction with the silica resins method (lane 2) or purification columns (lane 3). Lane 1 is the molecular weight marker in both cases. (A) Bone samples. (B) Industrial MBMs.

The next step consisted in evaluating which of the pretreatments would afford improved lysis of osteocytes and high-yield recovery of DNA from bones. Table 1 shows the parameters investigated and the average results obtained for each of them at the levels tested. DNA recovery was evaluated as DNA concentrations measured by fluorimetry, as indicated above. As seen in Table 1, all extraction procedures permitted the recovery of DNA from the bone samples, although different yields were obtained. In general terms, the highest recovery of DNA corresponded to the assays in which lysis was accomplished with 80 mL of proteinase K (10 mg/mL) for 3 h at 657C. Regarding the buffer used as extracting reagent in the pretreatments, DNA yields were lower when Milli-Q water was used, especially when the bone samples were treated at 607C overnight. By contrast, when 0.5 M EDTA was used as the extracting reagent at 607C, the recovery of DNA was significantly improved (Table 1). The use of EDTA has previously been considered by other authors for recovering DNA from ancient bone samples, although those studies addressed forensic samples [22, 31].

The introduction of 0.05% SDS, together with 0.5 M EDTA, as extracting reagent, afforded a high DNA extractability from osteocytes, especially when low levels of proteinase K or short lysis times were employed (Table 1). Despite this, the overall amount of DNA recovered was lower with respect to the assays in which the extracting reagent was 0.5 M EDTA alone and the temperature and time of extraction were 607C and 1 h, respectively (Table 1). Thus, the combination of 0.5 M EDTA and 0.05% SDS at 1007C for 1 h did not imply any advantage with respect to the pretreatment with EDTA alone. Treatment with ultrasound led to a 50% decrease in DNA recovery from the bone pellet even after treatment for a period as short as 5 min (Table 2). This observation,

together with the possible negative effect of ultrasound on DNA fragmentation, suggested that this pretreatment should not be further considered in our study.

As stated above, the most efficient pretreatment in terms of DNA recovery from bone pellets was pre-extraction of the bone material in 1 mL of 0.5 M EDTA at 607C for 1 h followed by centrifugation, after which the supernatant was separated from the pellet and the pellet was incubated at 657C for 3 h, in lysis buffer supplemented with 80 mL of proteinase K. The next step in this work aimed at evaluating the possible loss of DNA in the supernatants that might have been discarded during the pretreatment of bone meals with 0.5 M EDTA. Accordingly, bone meals were prepared by mixing bone fragments and wheat flour at a proportion of 10/90. These bone meals were pre-extracted in 0.5 M EDTA at 607C for 1 h. After spinning, the bone pellet and the supernatant were processed separately. Additionally, another assay was performed in which the spinning step was omitted, so the pellet and supernatant were processed together. The amounts of DNA recovered with each of the strategies are shown in Table 3. It may be seen that the combination of pellet and supernatant allowed us to double the recovery of DNA from the bone meal with respect to the processing of the pellet alone. This can be explained in terms of cellular lysis during the treatment with 0.5 M EDTA, this causing defragmentation of the bone matrix with the sub- sequent release of DNA to the extracellular medium. Accordingly, extraction of the supernatant together with the bone pellet afforded higher DNA yields with respect to the processing of the bone pellet alone. Moreover, as can also be observed in Table 3 pretreatment of the bone meal with 0.5 M EDTA at 607C for 1 h led to a 40% increase in DNA extractability as compared with the counterpart blank assay omitting this pretreatment.

3.2 DNA amplification

The second goal of this study was to evaluate whether the DNA obtained by the optimized extraction protocol described above could be amplified directly, and to check that none of the extracting reagents used in the optimization study, especially 0.5 M EDTA, was interfering with DNA polymerase in the amplification reaction. The results obtained clearly indicated that PCR products were obtained from all the assays (data not shown). Thus, pretreatment of bone samples with 0.5 M EDTA, combined or not with 0.05% SDS, did not affect DNA polymerase, all the extracts yielding amplification products. Likewise, the DNA extracts corresponding to animal meals made with bone powder and wheat flour at a 10/90 ratio were also subjected to PCR. Thus, DNA extracts obtained from the supernatant, the pellet, or from a combination

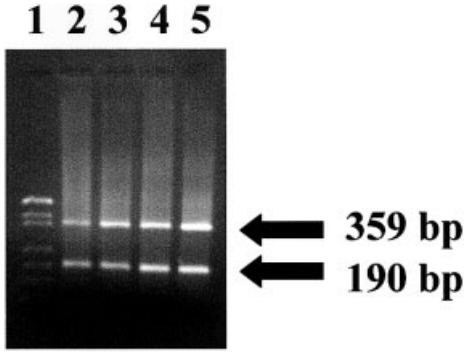

Figure 2. Agarose gel electrophoresis of the 359 bp and 190 bp PCR products. Pretreatment of bone meal samples was carried out in 0.5 M EDTA at 607C/1 h, extraction being performed by the purification column method (DNeasy, Qiagen). Lane 1, molecular weight marker; 2, supernatant of the pretreatment; 3, pellet of the pretreatment; 4, supernatant and pellet combined; 5, blank assay (supernatant and pellet combined) without pretreatment.

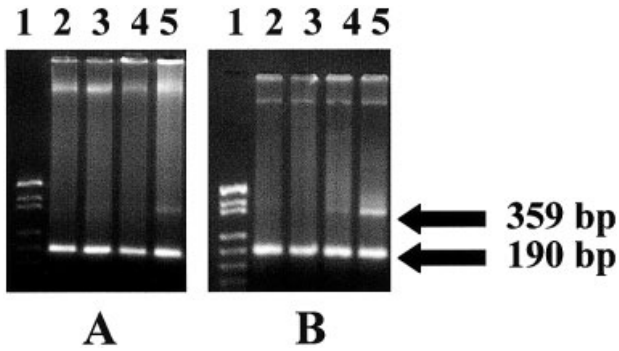

Figure 3. Sensitivity of the PCR protocol in the recovery and detection of DNA from bone remains in bone meals prepared by artificial contamination of wheat flour with either (A) animal bones or (B) industrial MBMs at different ratios. Lane 1, molecular weight marker; 2, 0% of bone/MBM; 3, 0.1% of bone/MBM; 4, 0.5% of bone/MBM; 5, 1% of bone/MBM.

of both of them combined were amplified using the Duplex-Master-Mix. The results are shown in Fig. 2 and clearly show that all the procedures tested yielded amplifiable DNA.

3.3 Sensitivity of the PCR method

The sensitivity of PCR in the detection of animal DNA in home-made bone meals and industrial MBMs was also evaluated and proved to be 1% and 0.5%, as can be observed in Figs. 3A and B, respectively. The high-temperature processing of industrial MBMs did not elicit any loss of sensitivity, DNA being detectable even at ratios as

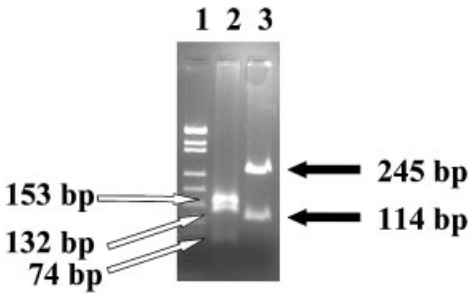

Figure 4. Species-specific electrophoretic analysis of bone samples by PCR-RFLP. Lane 1, molecular weight marker; 2, 359 bp PCR product cleaved with *Pal*I, which yields DNA fragments of 153 bp, 132 bp and 74 bp, specific of porcine; 3, 359 bp PCR product cleaved with *Mbo*I, which yields DNA fragments of 244 bp and 115 bp, specific of porcine.

low as 0.5%. As expected, the sensitivity of microscopy in the detection of bone material in animal meals was higher than that of PCR, allowing the detection of 0.1% of bone remains in home-made meals and industrial MBMs.

3.4 Species-specific cleavage of amplified DNA obtained from bone samples

Finally, the amplified DNA obtained from bone material could be successfully cleaved with endonucleases such as *Pal*I and *Mbo*I, an approach that is extremely useful for identification purposes, as can be seen in Fig. 4. Thus, Fig. 4 shows the RFLPs of DNA recovered from pig bone, showing DNA bands specific of this animal species, as it could be checked by comparison with the RFLPs obtained from a DNA standard from pig muscle (data not shown). No inhibition of the endonucleases by any of the reagents used in the pretreatment and in the extraction protocol was observed, being the amplified DNA ob- tained from pig bone successfully cleaved. Finally, and unlike other primers reported by other authors for bovine [32–34], porcine [35], ovine [35] and chicken [35] DNA, the primers used in this work, previously described by Meyer *et al.* [17], allow to obtain PCR products which, after digestion, deliver species-specific restriction pat- terns of any bone material from a wide variety of land animals in a single assay.

4 Concluding remarks

The optimized DNA extraction and purification procedure described in this work is recommendable because of its efficiency in DNA recovery from osteocytes. The highest extractability of DNA from osteocytes was obtained with pretreatment of the bone powder with 0.5 M EDTA at 607C for 1 h, followed by treatment with 800 mg of proteinase K for 3 h. When applied to home-made bone meals, this protocol allowed an overall increase in DNA extractability greater than 40% with respect to the extraction assay without pretreatment. The pretreatment described here also allowed successful amplification of target DNA sequences of the *cytb* mitochondrial gene in industrial MBMs. Since the primers used in this work are not specific to bovine species or indeed to any other single species, they allow the detection of bone or any other material from any land animal or fish in animal meals. Direct restriction analysis of the DNA amplification products with endonucleases renders this protocol species-speci- fic in the control of bone meals used for animal feeding.

The authors wish to express their thanks for the financial support received from Fundación Caixa Galicia, Santiago de Compostela, Spain.

Received November 7, 2001

5 References

[1] Wilesmith, J. W., Wells, G. A. H., Granwell, M. P., Ryan, J. B. M., *Vet. Rec*. 1998, *123*, 638–644.

[2] Bruce, M. E., Will, R. G., Ironside, J. W., McConnell, I., Drumond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Brikett, C., Cousens, S., Fraser, H., Bostock, C. J., *Nature* 1997, *389*, 498–501.

[3] Hill, A. F., Desbruslais, M., Joiner, S., Sidle, K. C. L., Growland, I., Collinge, J., Doey, L. J., Lantos, P., *Nature* 1997, *389*, 448–450.

[4] Momcilovic, D., Rasooly, A., *J. Food Prot.* 2000, *11*, 1602–1609.

[5] Kingombe, C. I. B., Lüthi, E., Schlosser, H., Howald, D., Kuhn, M., Jemmi, T., *Meat Sci.* 2001, *57*, 35–41.

[6] Rehbein, H., *Z. Lebensm. Unters. Forsch.* 1990, *191*, 1–10.

[7] Wolf, C., Rentsch, J., Hübner, P., *J. Agric. Food Chem.* 1999, *47*, 1350–1355.

[8] Piñeiro, C., Barros-Velázquez, J., Pérez-Martín, R. I., Martínez, I., Jacobsen, T., Rehbein, H., Kündiger, R., Mendes, R., Etienne, M., Jerome, M., Craig, A., Mackie, I. M., Jessen, F., *Electrophoresis* 1999, *20*, 1425–1432.

[9] Chen, F. C., Hsieh, Y. H., *J. AOAC Int.* 2001, *83*, 79–85.

[10] Piñeiro, C., Vázquez, J., Marina, A. I., Barros-Velázquez, J., Gallardo, J. M., *Electrophoresis* 2001, *22*, 1545–1552.

[11] Patterson, R. L. S., Jones, S. J., *Analyst* 1990, *115*, 501–506.

[12] Berger, R. G., Mageau, R. P., Schwab, B., Johnston, R. W., *J. AOAC Int.* 1988, *71*, 406–409.

[13] Meyer, R., Candrian, U., Lüthy, J., *J. AOAC Int.* 1994, *77*, 617–622.

[14] Allmann, M., Candrian, U., Höfelein, C., Lüthy, J., *Z. Lebensm. Unters. Forsch.* 1993, *196*, 248–251.

[15] Chikuni, K., Tabata, T., Saito, M., Monma, M., *Animal Sci. Technol.* 1994, *65*, 571–579.

[16] Meyer, R., Candrian, U., *Lebensm. Wiss. Technol.* 1996, *29*, 1–9.

[17] Meyer, R., Höfelein, C., Lüthy, J., Candrian, U., *J. AOAC Int.* 1995, *78*, 1542–1551.

[18] Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada, J., Shinmura, Y., *Meat Sci.* 1999, *51*, 143–148.
[19] Bartlett, S. E., Davidson, W. S., *BioTechniques* 1992, *12*, 408–411.
[20] Martínez, I., Yman, I. M., *Food Res. Int.* 1998, 31, 459–466.
[21] Simpson, T. A., Smith, R. J. H., *Laryngoscope* 1995, *105*, 28–34.
[22] Thomas, M. G., Moore, L. J., *BioTechniques* 1996, *22*, 402.
[23] Hagelberg, E., Clegg, J. B., *Proc. R. Soc. Lond.* 1991, *244*, 45–52.
[24] Pääbo, S., Higuchi, R., Wilson, A. C., *J. Biol. Chem.* 1989, *264*, 9707–9712.
[25] Waye, J. S., Willard, H. F., *Mol. Cell. Biol.* 1986, *6*, 3156–3165.
[26] Höss, M., Pääbo, S., *Nucleic Acids Res.* 1993, *21*, 3913–3914.
[27] Pääbo, S., in: *PCR Protocols: A Guide to Methods and Applications*, Academic Press Inc., San Diego, CA 1990, pp. 159–166.
[28] Hänni, C., Brousseau, T., Laudet, V., Stehelein, D., *Nucleic Acids Res.* 1985, *23*, 881–882.
[29] Cattaneo, C., Craig, O. E., James, N. T., Sokol, R. J., *J. Forensic Sci.* 1997, *42*, 1126–1135.
[30] Yang, D. Y., Eng, B., Dudar, J. C., Saunders, S. R., Waye, J. S., *Can. Soc. For. Sci. J.* 1997, *30*, 1–5.
[31] Lassen, C., Hummel, S., Herrmann, B., *Int. J. Leg. Med.* 1994, *107*, 152–155.
[32] Myers, M. J., Friedman, S. L., Farrell, D. E., Dove-Pettit, D. A., Bucker, M. F., Kelly, S., Madzo, S., Campbell, W., Wang, R. F., Paine, D., Cerniglia, C. E., *J. Food Prot.* 2001, *64*, 564–566.
[33] Tartaglia, M., Saulle, E., Pestalozza, S., Morelli, L., Antonucci, G., Battaglia, P. A., *J. Food Prot.* 1998, *61*, 513–518.
[34] Krcmár, P., Rencová, E., *J. Food Prot.* 2001, *64*, 117–119.
[35] Lahiff, S., Glennon, M., O'Brien, L., Lyng, J., Smith, T., Maher, M., Shilton, N., *Mol. Cell. Probes* 2001, *15*, 27–35.